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Ca²⁺-DEPENDENT PHOSPHATASE AND Ca²⁺-DEPENDENT ATPase ACTIVITIES IN PLASMA MEMBRANES OF EEL GILL EPITHELIUM—III. STIMULATION OF BRANCHIAL HIGH-AFFINITY Ca²⁺-ATPase ACTIVITY DURING PROLACTIN-INDUCED HYPERCALCEMIA IN AMERICAN EELS

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Abstract—1. Infusions of ovine prolactin for 10 days induced hypercalcemia in unfed American eels, *Anguilla rostrata* LeSueur, that tentatively was related to stimulation of branchial Ca²⁺-uptake mechanisms.

2. Analysis of ATPase activities in the plasma membranes of the branchial epithelium in prolactin treated eels showed a specific stimulation of high-affinity Ca²⁺-ATPase.

3. The results of this study form further evidence that the high-affinity Ca²⁺-ATPase activity represents the Ca²⁺-pump of the branchial epithelium.

INTRODUCTION

Fish can regulate their serum calcium levels with great precision (Bailey and Fenwick, 1975; Copp and Ma, 1978) and this ability must be attributed, at least in part, to the capacity of their gills to absorb calcium directly from their aquatic environment (Berg, 1968; Simmons, 1971; Simkiss, 1974; So and Fenwick, 1977; Payan *et al.*, 1981). Unfortunately, there is a paucity of information concerning the mechanisms of this branchial calcium uptake.

Recently we reported the simultaneous occurrence of non-specific phosphatase activity and high-affinity Ca²⁺-ATPase activity in American eel gill plasma membranes (Flik *et al.*, 1983, 1984). Further, we equated the heterogeneous non-specific phosphatase activity with the Ca²⁺-activated ATPase activity which has been reported present in the gills of many teleostean species and which has been described as related to calcium transport (Ma *et al.*, 1974; Fenwick, 1976; Moon, 1978; Fenwick, 1979; Ho and Chan, 1980; Doneen, 1981). However, the characteristics of this activity more closely resemble those of an alkaline phosphatase rather than a transport Ca²⁺-ATPase (Ghijsen *et al.*, 1980). On the other hand, we identified the branchial high-affinity Ca²⁺-ATPase as a calmodulin-sensitive ATPase that was stimulated by intracellular Ca²⁺-concentrations in the presence of excess Mg²⁺ (Flik *et al.*, 1984). These characteristics suggested that it was this latter activity which is associated with the branchial Ca²⁺-pump in fish.

The present study was directed towards testing the effect of ovine prolactin on both the Ca²⁺-dependent phosphatase and the high-affinity Ca²⁺-ATPase in American eel gill plasma membranes to determine which, if either, of the activities respond to prolactin treatment.

Prolactin is known to induce hypercalcemia in several species of fish (Pang *et al.*, 1978; Wendelaar Bonga and Flik, 1982), including American eel (Copp *et al.*, 1982). It was further reported that ovine prolactin enhanced calcium influx in perfused American eel gills (Ma and Copp, 1981). The rationale behind our present study was that if prolactin stimulates gill calcium absorption it should stimulate enzymic activities associated with active calcium transport.

MATERIALS AND METHODS

Adult female yellow eels, *Anguilla rostrata* LeSueur, with an average body weight of 1.7 kg were obtained from a commercial dealer in Québec City, Québec, Canada. The eels were held in running dechlorinated Ottawa tapwater (0.45 mM Ca²⁺, 12°C) with 16 hr of light alternating with 8 hr of darkness. The animals were not fed.

Hormone treatment

Ovine prolactin was kindly supplied by the Hormone Distribution Agency of the National Institutes of Health, Bethesda, MD and was administered continuously for 10 days by means of Alzet osmotic minipumps implanted intraperitoneally. The dosage was 0.1 U/g fish/day, dissolved in 0.05 N HCl. Controls received equivalent amounts of solvent. Ovine prolactin, at the doses used in this study,

Table 1. Effects of ovine prolactin on blood plasma mineral composition. Plasma mineral content is expressed in mM. Mean values \pm SD are given, with the number of animals in parentheses

	Controls (5)	Experimentals (4)
Na	141.5 \pm 14.1	135.8 \pm 6.9
K	1.89 \pm 0.06	1.84 \pm 0.19
Mg	0.91 \pm 0.05	0.50 \pm 0.12*
Ca	2.84 \pm 0.17	3.40 \pm 0.20*
P _i	1.35 \pm 0.13	1.12 \pm 0.20

* $P < 0.01$.

generally mimics homologous prolactin in its effects on osmoregulation and Ca^{2+} -metabolism of fish (Wendelaar Bonga and Van der Meij, 1980). At the end of the experiments the infusion rates of the pumps were checked by measuring the pump contents (maximum infusion duration of the pumps approximated 19 days at 12°C) and a maximum deviation of 10% was accepted. The stability of the hormone preparation was checked electrophoretically by comparison of freshly dissolved prolactin with prolactin recovered from the pumps at the end of the experiments. Silver stained electrophoretographs did not reveal any differences. Control and experimental animals were individually marked by fin clipping and were kept in the same tank during the experiment.

At the end of the experimental period the eels were anesthetized in a Tris-buffered (pH 7.4) MS222* solution and the blood was collected into heparinized tuberculin syringes by direct cardiac puncture. After centrifugation of the blood, total plasma Ca was estimated with a commercial Ca kit (Sigma, 586-A), Mg analyzed by atomic absorption spectrophotometry, and Na and K by flame emission spectrophotometry; inorganic phosphorus was analyzed by a modification of the method of Fiske and Subbarow (Fenwick, 1976).

Isolation of branchial plasma membranes and enzyme assays

Plasma membranes were isolated and assayed for protein, succinic dehydrogenase, Na^+/K^+ -ATPase, $\text{Ca} \sim \text{ATP}$ -phosphatase and high-affinity Ca^{2+} -ATPase activities as described previously (Flik *et al.*, 1983, 1984). Statistical analysis of the results was carried out applying Student's *t*-test (two-sided, $\alpha = 5\%$).

RESULTS

Blood plasma analysis

Prolactin treatment of eels increased plasma calcium levels significantly from 2.84 ± 0.17 mM in controls to 3.40 ± 0.20 mM ($P < 0.01$). Plasma total Mg levels were significantly decreased after this treatment ($P < 0.01$). No significant differences in plasma Na, K or P_i levels were observed (Table 1).

Enzyme analyses

Prolactin treated eels showed a statistically significant increase in high-affinity Ca^{2+} -ATPase specific activity (measured as the $1 \mu\text{M}$ Ca^{2+} induced $\text{Mg} \sim \text{ATP}$ hydrolysis). Neither Na^+/K^+ -ATPase nor $\text{Ca} \sim \text{ATP}$ -phosphatase activities were affected by this treatment. Succinic dehydrogenase specific activities in whole tissue homogenates and in the enriched plasma membrane fractions of control and prolactin treated fish did not differ significantly and were similar to values reported for untreated eels (Flik *et*

al., 1983). The amounts of total protein extracted from the gills and recovered in the plasma membrane fraction were not significantly different for control and experimental fish.

In these plasma membrane fractions calculated total high-affinity Ca^{2+} -ATPase activities were $13.4 \mu\text{mole P}_i/\text{hr}$ for controls and $21 \mu\text{mole P}_i/\text{hr}$ for prolactin treated eels (57% stimulation). Total Na^+/K^+ -ATPase activities were 157.2 and $170.8 \mu\text{mole P}_i/\text{hr}$ controls and experimentals, respectively. Ratios for total Na^+/K^+ -ATPase to high-affinity Ca^{2+} -ATPase were 11.7 for controls and 8.1 for prolactin treated eels, indicating a 44% stimulation of high-affinity Ca^{2+} -ATPase activity relative to Na^+/K^+ -ATPase activity in branchial plasma membranes after prolactin treatment (Table 2).

DISCUSSION

Prolactin and blood plasma mineral composition

Ovine prolactin induced hypercalcemia in fresh water yellow eels and this result agrees with earlier findings from killifish (Pang *et al.*, 1978), sticklebacks (Wendelaar Bonga and Greven, 1978), the tilapia *Sarotherodon mossambicus* (Wendelaar Bonga and Flik, 1982; Wendelaar Bonga *et al.*, 1983) and American eels (Ma and Copp, 1981). In addition, prolactin induced hypomagnesemia, a phenomenon also reported earlier for tilapia (Wendelaar Bonga *et al.*, 1983). In mammals such a hypomagnesemia is considered to be a direct effect of the concomitant hypercalcemia (Ebel and Guenther, 1980). Prolactin did not affect plasma Na, K or P_i levels, which indicates the specific nature of the hypercalcemic action of prolactin under these conditions. Ma and Copp (1981) have shown for American eels, the species used in this study, that this effect can be ascribed to stimulation by prolactin of Ca^{2+} -uptake in the gills.

Prolactin and branchial ATPase activities

Prolactin enhanced high-affinity Ca^{2+} -ATPase activities in the plasma membranes of eel branchial epithelium, which suggests that prolactin may influence Ca-metabolism by activating a Ca^{2+} -pump in the gills. Prolactin did not stimulate the non-specific $\text{Ca} \sim \text{ATP}$ -phosphatase activity. We take these observations as evidence that it is the high-affinity Ca^{2+} -ATPase and not the non-specific phosphatase which functions as a Ca^{2+} -transporting enzyme. Further, the recovery of branchial total Na^+/K^+ -ATPase, total $\text{Ca} \sim \text{ATP}$ -phosphatase and total protein were similar in prolactin treated and control eels. The absence of an effect on either the $\text{Ca} \sim \text{ATP}$ -phosphatase or the Na^+/K^+ -ATPase activities indicates that the action of prolactin on the gills is not of a general trophic character. This is also supported by the fact that the ratios of total Na^+/K^+ -ATPase to total high-affinity Ca^{2+} -ATPase differed between prolactin treated and control eels. The decrease of the ratio from 11.7 in the controls to 8.1 in prolactin treated eels suggested a specific induction of high-affinity Ca^{2+} -ATPase in branchial plasma membranes.

Interestingly, our results show some close simi-

*MS222, tricaine methanesulphonate; Tris tris(hydroxymethyl)aminomethane; $\text{Ca} \sim \text{ATP}$ -phosphatase, phosphohydrolase activity at 5 mM ATP and 2 mM Ca^{2+} .

Table 2. Effects of ten days infusion of ovine prolactin on plasma membrane ATPase and phosphatase activities in the gills. Specific activities are expressed as $\mu\text{mole P}_i \text{ h mg protein}^{-1}$. Mean values \pm SD are given with the number of animals in parentheses

	Controls (4)	Experimentals (4)	Statistical significance
High-affinity Ca ²⁺ -ATPase	2.56 \pm 0.50	4.94 \pm 0.58	$P < 0.01$
Na ⁺ /K ⁺ -ATPase	75.7 \pm 20.0	74.5 \pm 15.1	n.s.
Ca ~ ATP-phosphatase	81.2 \pm 10.0	80.3 \pm 7.4	n.s.

larities with data published by Ghijsen and Van Os (1982) on rat intestine. These authors reported that 1 α ,25(OH)₂ vitamin D₃ specifically stimulated a transport Ca²⁺-ATPase, that resembles the eel branchial Ca²⁺-ATPase closely, but not Na⁺/K⁺-ATPase in enterocytes from vitamin D₃-repleted rachitic rats; the ratios for Na⁺/K⁺-ATPase to transport Ca²⁺-ATPase activities amounted to 11.6 in the controls and 7.1 in 1 α ,25(OH)₂ vitamin D₃-repleted animals. Using rachitic rats, Pahuja and DeLuca (1981) showed that also prolactin directly influences intestinal Ca absorption in a way comparable to 1 α ,25(OH)₂ vitamin D₃; these authors concluded that the action of prolactin was independent of the vitamin D endocrine system.

In conclusion, it is our opinion that prolactin is involved not only in the control of branchial permeability for water and ions (Wendelaar Bonga and Van der Meij, 1981; Wendelaar Bonga *et al.*, 1983), but also in the regulation of the branchial Ca²⁺-pump. This contention may contribute new ideas to the discussion on the role of prolactin in hydromineral balance of fresh water teleosts.

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